Biochemical Characterization of the Midgut Serine Proteases of the Egyptian Cottonworm, *Spodoptera Littoralis* (Boisduval) and Their Interactions with Standard Protease Inhibitors

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***Abstract*—**Serine proteolytic activities in soluble protein extracted from the larval midgut of *Spodoptera littoralis* were studied using specific substrates and protease inhibitors. There are significant differences in serine activities in respect to larval development. The serine activities increase with instar development, reach each maximum at third instar larvae while the lowest serine activities are observed in the fifth instar larvae. The serine enzymes are stable at pH ranging from 6-11 with maximum activities at pH 10. Protease inhibitors negatively affect serine activities both *in vitro and in vivo.* Ingestion of trypsin inhibitor (SKTI) and chymotrypsin inhibitor (Chymostatin) cause alteration to larval development. In general, basic similarities among serine protease from *S. littoralis* and other lepidopteran insect digestive proteases in respect to optimum pH, substrate affinity and response to protease inhibitor are observed.

***Keywords*—**Serine Protease, Trypsine, Chymotrypsine, *Spodoptera littoralis*

I. Introduction

INSECT pests are one of the major biotic stresses to agricultural crops, responsible for sever crop reduction despite of the extensive use of chemical pesticide (Ferry *et al.*, 2004). Insects often inflict losses of 15 to 50% of the yield of some crops in various parts of the world and provide infection courts for various pathogens that inflict even greater damage. On the other hands plants are generally well suited to defend themselves against insect (Pilon *et al.*, 2006). The attack of pest insects on plants triggers the production of a serious of secondary metabolites; definisins, lectins, thioneines and inhibitors of serine proteases which all constitute the defensive arsenal of plants (Buchmanan *et al., 2002*). Protease inhibitors inhibit insect gut proteases by binding tightly to the active site, complex formation being essentially irreversible. The inability to utilize ingested protein and to recycle digestive enzymes results in critical amino acid deficiency, which affects the growth, development and survival of the herbivore (Chougule *et al.*, 2008). Efforts are being made to explore their use in developing insect resistance in crop plants (Boulter, 1993; Sharma *et al.*, 2000; Ferry *et al.*¸2004). Characterization of digestive proteolysis in different insect species would enable more choice of protease inhibitors in pest management strategies. This task becomes more complex in case for generalized feeders as they shown to be more adopted to several classes of inhibitors compared to specialized feeders (Broadway and Villani, 1995). Published data on lepidopteron insects revealed that they are mainly serine proteases (Purcell *et al*., 1992; Telang *et al*., 2005). The current study provides a biochemical analysis of the digestive gut proteolytic activity of *Spodoptera littoralis* larvae and the protease-protease inhibitor interaction.

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II. Materials and Methods

A. Materials

All enzyme standard substrates viz., N-a-benzoyl-DL-arginine-p-nitroanilide (BA*p*NA), N-a-benzoyl-DL-tyrosine-p-nitroanilide (BT*p*NA), sodium caseinate; standard inhibitors viz., Soybean Kunitz Trypsin Inhibitor (SKTI), Na-Tosyl-Lys-chloromethylketone (TLCK), Chymostatin , Tosyl phenylalanyl chloromethyl ketone (TPCK), phenylmethanesulfonylfluoride (PMSF); tris buffer and glycine buffer were procured from Sigma Chemical Co. (St. Louis, Mo, USA).

B. Insect cultures

A culture of *Spodoptera littoralis* originally obtained from the Plant Protection Research Center, Cairo, Egypt, was maintained on semi-artificial diet prepared as described by Kranthi (2005) at 27◦C, 60% RH).

C. Extraction of S. littoralis gut proteinases

Larvae at first to fifth instar stage were dissected out on ice and 10 food free midgut/ larval instar stage were taken out and gut enzyme extracts were prepared according to the method of Johnston *et a*l (1991) with some modifications. The midguts were homogenized in ice-cold 0.2 M glycine–NaOH buffer, pH 8 containing 2 mM DTT and 10% PVP (10 guts/ml buffer). The homogenates were kept for 2 h at 10 ◦C and centrifuged at 10000 rpm for 15 min at 4◦C. The resultant supernatant was used as a source of gut proteinases and stored at 20 ◦C for further use.

D. Protease activity assays

Total proteolytic activity was measured by sodium caseinate (5%) digestion using a modified method of Lee and Anstee (1995). Five grams of sodium caseinate was dissolved in 0.2 M Glycine-NaOH buffer, pH 9.6 by heating at 80 ^{0}C for 1-2 hr. Different equivalents (0.05 to 0.1 gut) of *S. littoralis* midgut extract were taken and mixed with 0.8 ml of sodium caseinate solution, which was thermoequilibrated with 0.2 ml of distilled water at 30 ^{0}C for 10 minutes. The reaction was stopped after 10 minutes by adding 30% tricholoro acetic acid (TCA). Blanks were run in which TCA was added before the addition of enzyme. Precipitated protein was removed by centrifugation at 8,000 rpm for 30 minutes and the absorbance was measured at 280 nm. Rate of proteolysis of sodium caseinate was expressed in units (1,000 x OD) of trichloroacetic acid soluble peptides released/min/mg protein (Geuden *et al*., 1998).

Activity of trypsin-like enzymes was measured using BA*p*NA as a substrate (Erlanger *et al.,* 1961). Different gut equivalents (0.05 to 0.1 gut) were mixed in 300 µl of 0.01 M Tris-Hcl buffer, pH 8.0 and incubated at 37 ^{0}C for 15 minutes. One milliliter of 1 mM BA*p*NA prepared in 0.01 M Tris-HCl containing 0.02 M $CaCl_2$ was added to start the reaction. The reaction mixture was incubated for 15 minutes at 37 ^{0}C and then the reaction was stopped by adding 200 µl of 30% acetic acid. The liberated *p*-nitroaniline was measured at 410 nm (Godbole *et al.,* 1991). (The extinction co-efficient for *p*-nitoraniline is ϵ=8800 cm^{-1}).

Chymotrypsin activity was measured using BT*p*NA as a substrate (Christeller *et al.,* 1989). Assays were carried out similar to trypsin-assay. Blanks were run in all assays. The enzyme activity was expressed as µmoles of *p*-nitroaniline hydrolyzed/min/mg protein using the formula:

Activity units =

$$\frac{\text{Abs 410 / min x 1000x ml of reeaction mixture}}{\text{Extinction coefficient of chromagen} - \text{mg protein in reaction mixture}}$$

Kinetic study assays were carried out measuring the initial rates of reaction with increasing substrate concentration for trypsin and chymotrypsin substrates.

E. Protein determination

Protein was determined according to the method of Lowry *et al*. (1951) where bovine serum albumin was used as a standard.

F. Stability of S. littoralis enzymes at different pHs

The stability of gut proteases at different pHs was measured as mentioned earlier. All experiments were carried out in triplicate at different pHs ranging from 2 to 11 using the following buffers at final concentrations of 50 mM: glycine–HCl for pH 2 and 3; Na-acetate–acetic acid for pH 4 and 5; phosphate buffer for pH 6 and 7; Tris–HCl for pH 8 , glycine–NaOH for pH 9 and 10 and CAPS buffer for pH 11 and 12. After 24 h incubation at each pH at room temperature, residual trypsin inhibitory activities were measured.

G. Effect of protease inhibitors on enzyme activity

Different concentrations (1 to 10.0 µg/ml) of Soybean Kunitz Trypsin Inhibitor (SKTI), Na-Tosyl-Lys-chloromethylketone (TLCK: trypsin inhibitor), Chymostatin (chymotrypsin inhibitor), Tosyl phenylalanyl chloromethyl ketone (TPCK**:** chymotrypsin inhibitor), phenylmethanesulfonylfluoride (PMSF: serine inhibitor) were used to determine the IC_{50} aginst proteases of *S. littoralis* midgut extract. All the inhibitors were mixed with 20 µl of midgut extract of the third instar larvae of *S. littoralis* and incubated at 37 ^{0}C for 10 minutes, before addition of substrate to start the reaction (Lee and Anstee, 1995). Residual activity was determined spectrophotometrically at 410 nm for trypsin and chymotrypsin and at 280 nm for general protease and results were expressed as IC_{50} relative to controls without inhibitor. All *in vitro* assays were carried out in triplicates and standard errors were calculated from the mean values

H. The effect of protease inhibitors on the larval development and protease activity of S. littoralis by feeding test

For feeding studies, the trypsin inhibitor (SKTI) and the chymotrypsin inhibitor (chymostatine) were incorporated into the artificial diet at different concentrations (w/w) of 0.5% while diet without added inhibitors was used as control diet. Starved third instar larvae were released into the rearing trays containing either control diet (or) inhibitor containing diet. Fresh diet was added as and when the larvae required or every alternate day. Larval weights were recorded at the same time every alternative day and data on larval mortality, percentage of pupation and pupal weight were also recorded. Three replications of 20 larvae each were used for each treatment and data was statistically analyzed.

To study the effect of inhibitors ingestion on the midgut protease activity, the midgut of the treated larvae were dissected out five days after feeding and protease enzymes were extracted and proteases activity were measured as described earlier.

III. RESULTS

The proteolytic activity of the midgut of first to fifth instar larvae of *S. littoralis* were assayed using synthetic substrates with respect to their specifies towards protease enzymes. Protein content and specific protease activity unite in different larval instar have been summarized in Table 1.

There were significant differences in the proteolytic activity at different larval stages. The proteolytic activity of *S. littoralis* increased with developing instars reaching its peak in the third instar larvae followed by gradually decrease at the fourth instar and reach its lower activity at the fifth instar larvae. Maximum proteolytic activity of 0.75± 0.06, 0.46±

0.06 and 2.18± 0.04 specific activity units for trypsin **(**BA*p*NA), chymotrypsin (BT*p*NA) and general protease (sodium caseinate) respectively were observed in the third instar larvae. The proteolytic activity was significantly lower at the fifth instar larvae as compared to other stages with specific activity units of 0.47± 0.03, 0.20± 0.01 and 1.01± 0.07 for trypsin, chymotrypsin and general protease respectively.

TABLE 1
PROTEIN CONTENT AND PROTEOLYTIC ACTIVITY OF *SPODOPTERA LITTORALIS* AT DIFFERENT LARVAL INSTARS

Larval instar	Protein content (mg/ml)	Proteolytic specific activity unit		
		Trypsin (BA*p*NA)	Chymotrypsin (BT*p*NA)	General protease activity (sodium caseinate)
First	12.03	0.64 ± 0.03^{b}	0.21 ± 0.01^{a}	1.32 ± 0.06^{b}
Second	17.04	0.68 ± 0.05^{bc}	0.24 ± 0.03^{a}	1.46 ± 0.06^{c}
Third	14.65	0.75 ± 0.06^{c}	0.46 ± 0.06^{c}	2.18 ± 0.04^{e}
Forth	12.58	0.67 ± 0.06^{bc}	0.38 ± 0.03^{b}	1.64 ± 0.05^{d}
Fifth	11.98	0.47 ± 0.03^{a}	0.20 ± 0.01^{a}	1.01 ± 0.07^{a}
C.V.		6.71	10.58	4.01
L.S.D.		0.079	0.058	0.110

Kinetic analysis of trypsin like and chymotrypsin like activity at pH 8.0 gave line reciprocal Michaelis-Menton (Lineweaver-Burk) plots, enable the estimation of *K*m , *V*max values (Table 2). The kinetic analysis revealed lower *K*m value and higher *V*max value for the trypsin specific substrate (BA*p*NA) compared with the chymotrypsin specific substrate (BT*p*NA). The *K*m values were 0.37 and 0.46 mM for BA*p*NA and BT*p*NA substrates, respectively. On the other hands, the *V*max value recorded for BApNA substrate was (3.57) which almost triple of that recorded for BT*p*NA substrate (1.11). These values indicate that trypsin specific substrate (BA*p*NA) has stronger affinity to enzyme and is more rapidly hydrolyzed when saturated compared to the chymotrypsin specific substrate (BT*p*NA).

TABLE II
KINETIC ANALYSIS OF THE MIDGUT PROTEASES OF THE THIRD INSTARS LARVAE OF SPODOPTERA LITTORALIS AGAINST SYNTHETIC SERINE PROTEASE SUBSTRATES

Substrate	*K*m (mM)	*V*max (µmol pNA released/min/mg protein
BA*p*NA (trypsin)	0.37	3.57
BT*p*NA (chymotrypsin)	0.46	1.11

The stability of the midgut protease of the third instar larvae of *S. littoralis* at different pHs ranging from 2 to 12 was measured using synthetic substrates (Fig 1). The general protease, trypsin and chymotrypsin enzymes were stable at pH ranging from 6 to 11. The proteolytic activities showed a continuous increase from pH 6 to pH 10 with an activity peak recorded at pH 10 followed by decrease in their activity at pH 11 while the proteolytic activities were at its lower level at pH 12 as the protease enzymes were unstable.

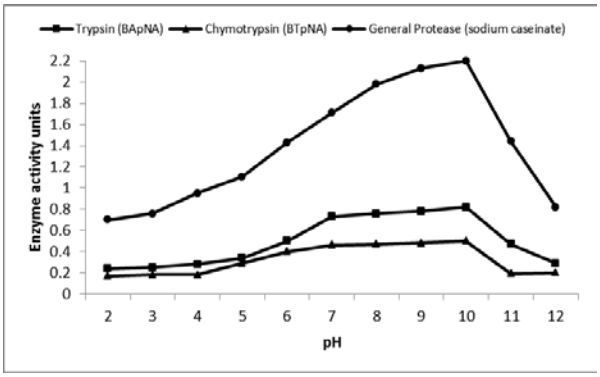

Fig.1. The effect of pH on the activity of serine proteases extracted from the midgut of third instar larvae of *Spodoptera littoralis.*

Effect of protease inhibitors on enzyme activity: Different concentrations (1 to 10.0 µg/ml) of standard specific protease inhibitors SKTI, TLCK, Chymostatin, TPCK and PMSF were used to determine the IC_{50} of proteases of *S. littoralis* midgut extract. All the assayed results showed linear inhibition of proteolytic activity with increasing of inhibitor concentration until saturation was achieved. Calculated values of IC_{50} are presented in Table 3. Inhibition of trypsin activity using the trypsin specific inhibitors SKTI and TLCK gave IC_{50} values of 3±0.11 and 9±0.76 µg/ml, respectively. Maximum trypsin inhibition of more than 95 % was observed at concentration of 15 and 35 µg/ml for SKTI and TLCK, respectively. However, no trypsin inhibition was achieved using the chymotrypsin inhibitors, Chymostatin and TPCK. The general serine proteinase inhibitor, PMSF caused trypsin inhibition with IC_{50} value of 8.3 ± 0.32 µg/ml and cause maximum trypsin inhibition of more than 95% at concentration of 55 µg/ml.

TABLE III
THE IN VITRO EFFECT OF DIFFERENT PROTEASE INHIBITORS ON ACTIVITY OF SERINE PROTEASES EXTRACTED FROM THE MIDGUT OF THIRD INSTAR LARVAE OF SPODOPTERA LITTORALIS.

Inhibitor	IC_{50} (µg/ml)	
	Trypsin (BA*p*NA)	Chymotrypsin (BT*p*NA)
SKT1 (trypsin specific)	3 ± 0.11	> 100
TLCK (trypsin specific)	9 ± 0.76	> 100
Chymostatin (chymotrypsin specific)	> 100	2.56 ± 0.32
TPCK (chymotrypsin specific)	> 100	1.89 ± 0.24
PMSF (serine proteinase specific)	8.3 ± 0.32	7.4 ± 0.49

Inhibition of chymotrypsin activity using the chymotrypsin inhibitors Chymostatin and TPCK gave IC_{50} values of 2.56 ± 0.32 and 1.89 ± 0.24 µg/ml, respectively. Maximum chymotrypsin inhibition of more than 95 % was observed at concentration of 10 and 8 µg/ml for Chymostatin and TPCK, respectively. However, no chymotrypsin inhibition was achieved using the trypsin inhibitors, SKTI and TLCK. The serine proteinase specific inhibitor, PMSF cause chymotrypsin inhibition with IC_{50} value of 7.4 ± 0.49 µg/ml and cause maximum chymotrypsin inhibition of more than 95% at concentration of 45µg/ml.

The effect of protease inhibitor on the larval development and protease activity of S. littoralis by feeding test:

The antimetabolic effects of the standard protease inhibitor were tested against the third instar larvae of *S. littoralis* by incorporating the trypsin inhibitor, SKTI and the chymotrypsin inhibitor, Chmostatin into its artificial diet at a level of 0.5%. The mean larval weight, larval mortality, pupation percentage and mean pupal weight were recorded and presented in Table 4. The larvae fed on the artificial diet without any inhibitor gained 398.25 ±24.58 mg after 9 days of feeding which was significantly higher than the larvae fed on diet containing either trypsin inhibitor (SKT1) or chymotrypsin inhibitor (chymostatin). The negative effects of inhibitors on the larvae growth rate started to appear three days after feeding. The larvae fed on diet containing trypsin inhibitor (SKT1) gained lower weight (220.8 ±34.02 mg) compared with that fed on diet containing chymotrypsin inhibitor 280.6±46.01 after 9 days of feeding. Feeding larvae on diet containing SKTI caused 53.33% mortality which was significantly higher compared to diet containing chymostatin (36.66%). The pupation percentage was negatively affected by adding inhibitors to larvae diet compared to control. The decline in pupation percentage was higer in case of trypsin inhibitor (46.66%) compared to chymotrypsin inhibitor (66.33%). The mean pupal weight in control treatment was (260.25±3.15 mg) which was significantly high compared to trypsin inhibitor (198.45±7.13 mg) and chymotrypsin inhibitor (210.42± 6.90 mg) treatments. However, there was no significant difference in the pupal weight between SKTI and chymostatin treatments.

TABLE IV
DEVELOPMENT OF THE THIRD INSTAR LARVAE OF SPODOPTERA LITTORALIS REARED ON THE DIET CONTAINING COMBINATIONS OF CHICK PEA AND STANDARD PROTEASE INHIBITOR.

Treatments	Mean fresh weight of the larvae (mg ± SD)					Larval mortality %	Pupation %	Mean pupal weight (mg ± SD)
	D1*	D3	D5	D7	D9			
Control (Chick pea only)	24.20[a] ±0.95	135.15[a] ±1.35	246.30[a] ±1.34	338.6[a] ±8.70	398.25[a] ±24.58	6.66	93.33	260.25[a] ±3.15
SKT1 (0.5%) Trypsin inhibitor	25.40 a ±0.36	114.78 b ±2.56	164.5 c ±9.40	180.86 c ±29.14	220.8 c ±34.02	53.33	46.66	198.45b ±7.13
Chymostatin (0.5%) chymotrypsin inhibitor	25.30 a ±0.88	120.49 b ±3.45	198.66 b ±11.09	240.03 b ±32.58	280.6 b ±46.01	36.66	66.33	210.42 b ±6.90
L.S.D.	1.54	8.57	13.35	30.34	36.02			13.52

Means in a column followed by the same letter are not significantly different
* The first day of treatment

The effect of inhibitor ingestion on the larval midgut protease activity was tested by measuring the trypsin and chymotrypsin activity on the midgut of treated larvae after 5 days of feeding. Ingestion of the trypsin inhibitor (SKT1) caused 42.67% reduction in the midgut trypsin activity of the treated larvae compared to control. However ingestion of the chymotrypsin inhibitor (chymostatin) caused 26.09% reduction in midgut chymotrypsin activity (Fig. 2).

IV. DISCUSSION

For an efficient management of pest control through proteinase inhibitor transgene it is imperative to characterize the protease enzyme present in the pest midgut. The two major proteinase classes present in the digestive systems of phytophagous insect are the serine and cysteine proteases (Haq *et al*., 2004). Srinivasan *et al*., 2006 carried out a study on the midgut enzymes of various pests belong to Lepidoptera and reported that serine proteases dominate the larval gut environment and contribute to about 95% of the total digestive in Lepidoptera. In the current study the presence of eerine proteases, trypsin likes enzymes and chymotrypsine like enzymes in *S. littoralis was* clearly demonstrated by the hydorlysed of the trypsin specific substrate BApNA and the chymotrypsine specific substrate BT*p*NA respectively and also by the inhibition of enzymes activity by using the trypsin specific inhibitors (SKT1, TLCK), chymotrypsin specific inhibitors (Chymostatin, TPCK) and the serine inhibitor (PMSF).

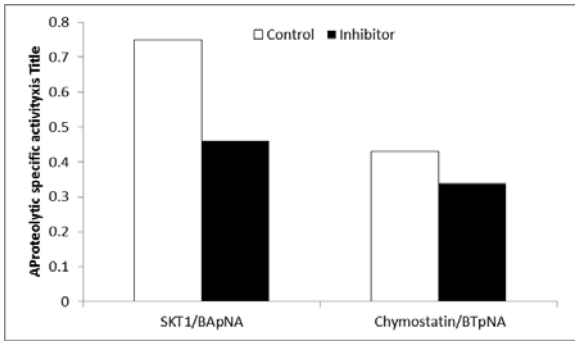

Fig. 2. The effect of inhibitor ingestion on the activity the activity of midgut serine proteases of third instar larvae of *Spodoptera littoralis.*

The midgut proteolytic activity of *S. littoralis* in regards to the larval stages was measured. The proteolytic activity of *S. littoralis* increased with developing instars reaching its peak in the third instar larvae followed by gradually decrease at the

fourth instar and reach its lower activity at the fifth instar larvae. This decline in protease activity of the fifth larval instar might have been resulted from great decrease or lower synthesis of digestive protease produced by the decrease of food intake when a larva is about to pupate. Therefore, the diminution in enzymatic activity could be related to anatomical and physiological modification of the larvae gut (Alarcon *et al.*, 2002). These results are in consonance with that of Satheesh and Murugun (2012) who reported maximum protease activity in the third and fourth larval instar of *S. littoralis* corresponding to ninth day of larval development. Sharma (2013) reported significant differences in the proteolytic activity of different stages of *S. littoralis*. He observed maximum proteolytic activity at the second instar larvae while the proteolytic activity was at its lower at the late fifth instar larvae.

The kinetic parameters for trypsin like enzyme and chymotrypsin like enzyme indicated that trypsin specific substrate (BA*p*NA) have stronger affinity to enzyme and is more rapidly hydrolyzed when saturated compared to the chymotrypsin specific substrate (BT*p*NA) as lower *K*m value and higher *V*max value were observed for trypsin like enzyme compared to chymotrypsin like enzyme.

The general protease, trypsin and chymotrypsin enzymes of *S. littoralis* were found to be stable at wide range of pH from 6 to 11 with maximum activity recorded at pH 10 while enzymes were not stable at pH 12. Lee (1993) reported that the pH of *S. littoralis* gut is ranging from pH 8 to 10. He also reported that trypsin enzyme and chymotrypsin enzyme are stable from pH 4 to 12 with maximum activity at pH 8 -10. Dorrah, 2004 reported that the pH optimum for trypsin like enzyme from *S. littoralis* is pH 11 while pH 10 was optimum for chymotrypsin like enzyme.

Two standard trypsin specific inhibitors SKTI, TLCK and two specific chymotrypsin inhibitors Chymostatin, TPCK and the serine inhibitor, PMSF were tested for their activities against *S. littoralis* midgut protease. SKTI and TLCK inhibited the trypsin like enzyme while SKTI was stronger than TLCK. The chymotrypsin specific inhibitors, Chymostatin and TPCK inhibited chymotrypsin like enzyme activity while chymostatin was less stronger compared to TPCK. These Four inhibitors has also shown the similar effects against trypsin like and chymotrypsin like enzymes of many other lepidopteran larvae (Dorrah, 2004, Telang *et al*., 2005, Chougule *et al.*,2008). The general serine inhibitor, PMSF could inhibit both trypsin and chymotrypsin like enzyme while PMSF was quite stronger against chymotrypsin like enzyme compared to trypsin like enzyme which may be due to high relative affinity of PMSF inhibitor with bovine chymotrypsin compared with bovine trypsin (Lee, 1993)

Feeding *S. littoralis* larvae on diet containing 0.5% SKTI or 0.5% Chymostatin caused reduction in larval weight, pupation and pupal weight and increased larval mortality. The negative effect caused by the trypsin inhibitor, SKTI on larval development was more strongly than that caused by the chymotrypsin inhibitor, chmostatin. Following the same trend, ingestion of SKTI by *S. littoralis* larvae resulted in 42.67% reduction in the midgut trypsin activity; however ingestion of the chymotrypsin inhibitor (chymostatin) caused 26.09% reduction in midgut chymotrypsin activity. Dorrah, 2004 reported that serine protease activities were significantly inhibited *in vitro* and *in vivo* by soybean trypsin inhibitor (SKTI) in *S. littoralis* larvae. The incorporation of different concentrations of SKTI into an artificial diet, consumed by the larvae, caused variable *in vivo* inhibitory effects. These effects were more prominent in the fourth instar; however, in the fifth instar the proteases activity was recovered again. The ingestion of dietary SKTI also caused reduction in the weight gain and survival of treated larvae. The negative effects on larval development and midgut enzymes activity caused by incorporating trypsin inhibitor and chymotrypsin inhibitor in insect diet were demonstrated by other authors in different lepidopteran species (lee, 1993, Gatehouse *et al*., 1997, 1999, Telang *et al*., 2005, Chougule *et al.*, 2008).

In conclusion, the results of this study indicate a basic similarity among the proteases from *S. littoralis* and other lepidopteran insect digestive proteases in respect to optimum pH, substrate affinity and response to different protease inhibitor. The specify of inhibitors toward *S. littoralis* midgut protease (trypsin like and chymotrypsin like enzyme) revealed the need to select protease inhibitors (PIs) that would inhibit both trypsin like and chymotrypsin like proteases to achieve sustainable crop protection and case to case studies on insect proteases and their biochemical characterization are required for better selection of PIs for inhibition of diverse range of insect gut proteases (Ferry *et al*., 2004; Telang *et al.*, 2005).

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